

Apoptosis Induction by Activator Protein 2 α Involves Transcriptional Repression of Bcl-2*[§]

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Activator protein 2 α (AP-2 α) induces cytotoxicity by inducing cell cycle arrest and apoptosis. In this study we investigated the mechanism of apoptosis induction by AP-2 α . We found that AP-2 α induced apoptosis efficiently in cells treated with benzyloxycarbonyl-IETD-fluoromethyl ketone or FADD-silenced cells but failed to do so in benzyloxycarbonyl-LEHD-fluoromethyl ketone-treated or apoptosis protease activation factor-1 (Apaf1)-silenced cells, suggesting the central role of mitochondria in AP-2 α -induced apoptosis. In good correlation, cells overexpressing AP-2 α showed a reduction in mitochondrial membrane potential ($\Delta\psi_m$), cytochrome *c* and Smac/DIABLO release into cytosol, and Bax translocation into mitochondria. We found that the pro-apoptotic protein Bax is important for AP-2 α -induced apoptosis as adenovirus AP2 failed to induce apoptosis in HCT116 Bax^{-/-} cells. However, we found the IAP (inhibitor of apoptosis) inhibitor Smac/DIABLO may have a limited role in AP-2 α -induced apoptosis as we found the IAP member Survivin down-regulated by AP-2 α . Although the total Bax level remains unaltered, we found a time-dependent increase in the activated form of Bax in adenovirus AP2-infected cells. In addition, we show that AP-2 α transcriptionally represses Bcl-2 by binding to its promoter both *in vitro* and *in vivo* and that this is essential for AP-2 α -induced apoptosis as ectopic expression of Bcl-2 efficiently inhibited apoptosis induced by AP-2 α . Furthermore, we show that chemotherapy-induced endogenous AP-2 α down-regulates Bcl-2 and induces apoptosis in an AP-2 α -dependent manner. Moreover, we demonstrate that inhibition of okadaic acid or staurosporine-sensitive pathways in AP-2 α overexpressing breast cancer cells resulted in AP-2 α -dependent apoptosis induction. These results suggest that AP-2 α induces apoptosis by down-regulating Bcl-2 and utilizing a bax/cytochrome *c*/Apaf1/caspase 9-dependent mitochondrial pathway.

Activator protein 2 α (AP-2 α)³ is a sequence-specific DNA binding transcription factor that is required for normal growth and morphogenesis (1–3). AP-2 α has a conserved C-terminal DNA binding motif with an integral helix-span-helix homodimerization motif and a less-con-

served proline and aromatic amino acid-rich helix transactivation domain near the N terminus and binds to a consensus DNA sequence, 5'-GCCNNNGGC-3' (4–8). AP-2 α has been shown to regulate many genes involved in variety of biological functions (9).

Several lines of evidences indicate that AP-2 α may act as a tumor suppressor gene. AP-2 α gene is located in chromosome position 6p22, a region of frequent loss of heterozygosity in breast and other cancers (10). Diminished AP-2 α function has been correlated with N-Ras oncogene-mediated transformation (11). The functions of AP-2 α have been shown to be regulated by SV40 T antigen and adenovirus E1A oncoproteins (1, 12). In addition, reduced or loss of AP-2 α expression has been reported in human cancers of breast, ovary, colon, skin, brain, and prostate (13–20). In good correlation, expression of dominant negative mutant AP-2 α resulted in increased invasiveness and tumorigenicity (21).

Overexpression of AP-2 α by transient transfection into cultured cells has been shown to induce p21^{WAF1/CIP1} and inhibit cellular DNA synthesis and colony formation (22). Significant correlation between AP-2 α expression and p21^{WAF1/CIP1} has been observed in breast cancer, colorectal cancer, and malignant melanoma (15, 18, 23). The growth inhibitory activity of AP-2 α has also been shown to be mediated through direct interaction with p53 (24). Results from our laboratory suggest that adenovirus-mediated overexpression of AP-2 α inhibits growth of cancer cells by inhibiting cellular DNA synthesis and inducing apoptosis (25). Furthermore, our recent work establishes that AP-2 α is induced in cancer cells upon treatment with chemotherapeutic drugs, which contributes to chemosensitivity because the simultaneous inhibition of AP-2 α by siRNA increases the chemoresistance. In addition, the re-expression of epigenetically silenced AP-2 α in breast cancer resulted in enhanced chemosensitivity and loss of tumorigenicity upon chemotherapy in an AP-2 α -dependent manner (26). These results point out the importance of apoptosis induction by AP-2 α for its functions, particularly the role in chemosensitivity.

The molecular mechanism by which AP-2 α induces apoptosis is not known. In the present study we have analyzed the pathways and various molecules involved in AP-2 α -induced apoptosis. We found that AP-2 α -induced apoptosis requires primarily the mitochondrial pathway involving a bax/cytochrome *c*/Apaf1/caspase 9-dependent mechanism. We also found that AP-2 α binds to the Bcl-2 promoter leading to its transcriptional down-regulation and is essential for the apoptosis induction by AP-2 α . In addition, we provide evidence that the overexpressed AP-2 α (perhaps functionally inactive) in certain breast cancer cells can be made functional by inhibiting survival signaling pathways sensitive to okadaic acid and staurosporine.

EXPERIMENTAL PROCEDURES

Plasmids, Adenoviruses, siRNA, Cell Lines, and Culture Conditions—pCEP4/AP-2 α was generated by cloning AP-2 α cDNA as a BamHI/HindIII fragment originally derived from pSG5/AP-2 α , which was kindly provided by Dr. P. Kannan (Metro Health Medical Center, Case

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1 and S2.

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³ The abbreviations used are: AP-2 α , activator protein 2 α ; FADD, Fas-associated death domain; IAP, inhibitor of apoptosis; Apaf1, apoptosis protease activation factor-1; PARP, poly(ADP-ribose) polymerase; m.o.i., multiplicity of infection; MOMP, mitochondrial outer membrane permeabilization; fmk, fluoromethyl ketone; z-, benzyloxycarbonyl; si, small interfering; Ad, adenovirus; GST, glutathione S-transferase; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FACS, fluorescence-activated cell sorter; WT, wild type; 2N, diploid.

AP-2 α Represses Bcl-2 to Induce Apoptosis

Western Reserve University). pD5neo-bcl-2 was obtained from Dr. S. Pervaz Hussain, NIH, through Dr. Apurva Sarin, National Center for Biological Sciences, Bangalore. Human cancer cell lines SW480, HCT116 WT, and H460 were described previously (25, 27). HCT116 Bax^{-/-} cells were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University) (28). Ad-LacZ and Ad-AP2 lack both E1A and E1B but carry β -galactosidase and AP-2 α , respectively (25). FADD and Smac/DIABLO-specific siRNA were obtained from Dharmacon as siGENOMETM SMARTpool reagent, which contains a pool of four different double-stranded RNA oligonucleotides (siRNA) directed against the particular gene. Apaf1 siRNA is described previously (29) and was also bought from Dharmacon as a duplex. Lamin siRNA was purchased as siGLO Lamin A/C siRNA (human/mouse/rat) from Dharmacon. The cells were transfected using Oligofectamine (Invitrogen) at a 100 nM concentration of siRNA. Bcl-2 promoter construct, Bcl-2prom (1.9 kilobases)-Luc, (-1291 to +610) was a kind gift of Dr. Fritz Aberger, University of Salzburg, Salzburg, Austria (30). GST-AP-2 α DNA binding domain was constructed by cloning the region of AP-2 α between amino acids 165 and 437 into TA cloning vector from which it was released as BamHI and then fused in-frame with GST in pGEX-5X-1.

Immunoprecipitation, Western Blot Analysis, Bromodeoxyuridine Incorporation Assay, and Cell Cycle Analysis—Conformationally changed Bax is immunoprecipitated using Bax conformation-specific antibody (6A7; Sigma) as described previously (31). Western blot analysis was performed as described previously (25) with mouse anti-human PARP monoclonal (Ab-2; Oncogene), rabbit anti-human AP-2 polyclonal (sc-184; Santa Cruz), goat anti-human actin polyclonal (sc-1616, Santa Cruz; Ab-1, Oncogene) antibodies, β -actin (AC-15, Sigma), NADH-ubiquinone oxidoreductase (Complex I, 20C11; Molecular Probes), mouse anti-human Caspase 8 (Ab-3; Oncogene), rabbit anti-human caspase 9 (Ab-2; Oncogene), mouse anti-human caspase 3 (9662; Cell Signaling), Bax (N-20; Santa Cruz), Smac/DIABLO (PC547; Oncogene), and cytochrome *c* (sc-13560; Santa Cruz). Bromodeoxyuridine incorporation and cell cycle analysis were done as described previously (25).

RNA Isolation, Semiquantitative RT-PCR, and Quantitative Real-time RT-PCR—Total RNA was extracted from tissue culture cells by TRIzol method (Invitrogen) according to the manufacturer's instructions. The RNA samples were quantified using a spectrophotometer and visualized on a Tris borate-EDTA gel for quality assurance. RT-PCR was carried out using a two-step strategy (32); cDNA was generated using reverse transcription kit (Promega) in the first step and then using gene-specific primer sets, and PCR was carried out with cDNA as templates. Glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) is used for internal normalization. The sequences of the sense (S) and antisense (AS) primers used for RT-PCR are: GAPDH (S, 5'-TTGTCAAGCTCATTTCCTGG-3', and AS, 5'-TGATGGTACATGACAAGGTGC-3'); AP-2 α (S, 5'-GCATCCAGAGCTGCTTGACC-3', and AS, 5'-GAGCCTCACTTCTGTGCTTCTC-3'); lamin A/C (NM_170708 (S, 5'-ACTCATCCCA-GACAGAGGGTG-3', AS, 5'-ATTGGACTTGTTGCGCAGC-3'); Apaf1 (S, 5'-TCACGTTCAAAGGTGGCTGAT-3', AS, 5'-CGTCT-TATATGGTCAACTGCAAGG-3'); FADD (S, 5'-TCAAGCTGCG-TTTATTAATGCC-3', AS, GACACGGTTCCAACCTTCCAAC-3'); Smac/DIABLO (S, GCGCGATCCATGGCGGCTCTGAAGAGTTG, AS, AGCTCTCTAGACTCAGGCCCTCAATCCTCA-3').

For PCR, template was melted at 95 °C for 5 min followed by amplification for 25 cycles at 95 °C, melting for 30 s at 58 °C, annealing for 1 min, a 72 °C extension for 1 min, and a final extension for 10 min. The quantitative real time RT-PCR was carried essentially as described before (32). Sequences for PCR primers for AP-2 α and GAPDH are given above. Sequences of PCR primers for Survivin and Bcl-2 are the

following: Survivin (S, 5'-CAGATTTGAATCGCGGGACCC-3', AS, 5'-CCAAGTCTGGCTCGTTCTCAG-3'); Bcl-2 (S, 5'-CCTGTGGAT-GACTGAGTACC-3', and AS, 5'-GAGACAGCCAGGAGAAATCA-3').

MTT Assay and Stable Colony Suppression Assay—MTT assay was carried out as described previously (25). For colony suppression assay, the cells were transfected with pCEP4 and pCEP4/AP-2 α plasmids and selected on hygromycin (250 μ g/ml).

Mitochondrial Membrane Potential ($\Delta\psi_m$)—Alteration in mitochondrial membrane potential ($\Delta\psi_m$) were analyzed by flow cytometry using the $\Delta\psi_m$ -sensitive dye JC-1 (5,5',6,6' tetrachloro-1,1',3,3' tetraethylbenzimidazolcarbocyanine iodide; Calbiochem) (33). Briefly, cells were harvested, washed once in phosphate-buffered saline (PBS), resuspended in 1 \times PBS, and incubated with 1 μ M JC-1 at 37 °C for 10 min. Stained cells were then washed once in phosphate-buffered saline and analyzed by flow cytometry. JC-1 monomers emit at 527 nm (FL-1 channel), and "J-aggregates" emit at 590 nm (FL-2 channel). Valinomycin-treated cells were used for compensation (FL-1-FL-2), and a cytofluorometric profile from these cells defines the 590-nm cutoff for untreated *versus* treated cells.

Generation of Bcl-2 Stable Cell Lines—SW480 cells were transfected with Bcl-2 plasmid (pD5neo-bcl-2) and selected for G418 resistance (800 μ g/ml). Medium was changed periodically every third day. Resistant colonies were cloned and screened for Bcl-2 expression by Western blot analysis. Positive clones were checked for clonal purity and used for further experiments.

Preparation of Mitochondrial and Cytosolic Fractions—Cells were lysed in buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and a mixture of protease inhibitors, which contained antipain, leupeptin, pepstatin A, and chymostatin). Lysis buffer was added and incubated for 30 min on ice. In addition the cells were homogenized by 30 strokes in a 22-gauge needle. Homogenates were centrifuged at 750 \times g for 10 min at 4 °C. Supernatants were centrifuged at 10,000 \times g for 15 min at 4 °C to collect the mitochondrial pellets. The supernatant after mitochondrial pelleting was used as a cytosolic fraction.

Luciferase Assay—Transfections were carried out either by using Escort III (Sigma) as per the manufacturer's recommendations. In all transfections 1 μ g of pCMV-LacZ DNA was added to normalize the transfection efficiency variation between samples. Luciferase and β -galactosidase assays were performed as described previously (22).

Electrophoretic Mobility Shift Assay—The DNA binding domain of AP-2 α (165–437) is purified as GST-AP-2 α fusion protein and used for electrophoretic mobility shift assay. Double-stranded probes were end-labeled by the T4 polynucleotide kinase using [γ -³²P]ATP. 20,000 cpm of ³²P-labeled probe and 1 μ g/reaction of poly(dI-dC) was added in a 20- μ l reaction volume (containing 10 mM Tris-HCl, pH 7.9, 60 mM KCl, 4 mM MgCl₂, 0.1 mM EDTA, 50 μ g/ml bovine serum albumin, 0.2% Nonidet P-40, and the competing oligonucleotides) for 30 min on ice. Samples were loaded onto 0.5 \times Tris borate-EDTA, 5% acrylamide gel and electrophoresed at 4 °C. The following oligonucleotides and their complements were synthesized and annealed: Bcl-2wt, 5'-CTAATTTT-TACTCCCTCTCCCCCGACTCCTGA-3'; Bcl-2m, 5'-CTAATTTT-TACTCCTATTCCCAAAGACTCCTGA-3'; SV40-AP2wt, 5-GATC-CAAAGTCCCCAGGCTCCCCAG-3'; SV40-AP2m, 5-GATCCAAAG-TCTCCGAATTCTCGAG-3'.

Chromatin Immunoprecipitation Assay—Chromatin immunoprecipitations were performed as described previously (34). Briefly, subconfluent SW480 cells that were infected with Ad-AP2 for 24 h were treated with formaldehyde at a final concentration of 1% for 7 min at

room temperature. Chemical cross-linking was terminated by the addition of glycine to a final concentration of 0.125 M followed by additional incubation for 5 min. After a wash with cold phosphate-buffered saline, cells were suspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, and 10 μ g/ml antipain, leupeptin, pepstatin A, and chymostatin) and kept at 4 °C for 10 min. The chromatin was then sonicated as described (34) so that the total DNA is sheared to 200–1000 bases in length. After centrifugation, the supernatant was diluted 10-fold with dilution buffer (16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, and 10 μ g/ml antipain, leupeptin, pepstatin A, and chymostatin). Diluted chromatin was precleared with protein A-agarose beads saturated with bovine serum albumin (1 mg/ml) and salmon sperm DNA (500 μ g/ml) and then incubated overnight at 4 °C using anti-AP-2 (C-18, Santa Cruz) or anti-mouse lamin B (Oncogene) and immunoprecipitated with protein A-agarose beads. The beads were extensively washed, and then chromatin was eluted from beads by incubation in elution buffer (0.1 M NaHCO₃, 1% SDS) for 2 h. Cross-links were then reversed by overnight incubation at 65 °C in elution buffer containing 300 mM NaCl and 30 μ g of RNase A/ml. A 20% equivalent amount of diluted chromatin was similarly processed without immunoprecipitation and noted as “input” afterward. DNA samples were then purified by phenol-chloroform extraction and ethanol precipitation and further analyzed by PCR for the region of interest. The primers used to amplify a 173-bp fragment spanning –129 to +44 of Bcl-2 promoter are as follows: upstream primer, 5-CGGTTGGGATTCCTGCGGATT-3', and downstream primer, 5-AATTGCATAAGGCAACGATCCC-3'.

RESULTS

AP-2 α Induces Apoptosis through an Intrinsic Pathway—AP-2 α overexpression is highly cytotoxic to cancer cells as it inhibits cellular DNA synthesis leading to G₁ cell cycle arrest and induces apoptosis. To study the relative role of intrinsic versus extrinsic pathway in AP-2 α -induced apoptosis, we examined the cells infected with AP-2 α adenovirus (Ad-AP2) for activated forms of major effector caspases and their importance. In all our experiments where recombinant adenoviruses were used, a control adenovirus expressing β -galactosidase (Ad-LacZ) was also used. Infection of H460 human lung carcinoma cells with Ad-AP2 resulted in efficient induction of apoptosis as seen by the appearance of cells with less than a 2N amount of DNA, representing apoptotic cells, by 48 h of virus infection (Fig. 1A) and efficient PARP cleavage in a time-dependent fashion (Fig. 1B). AP-2 α overexpression activated caspase 3, 8, and 9 as seen by the appearance of cleaved forms of these caspases in Ad-AP2- but not in Ad-LacZ-infected cells (Fig. 1C). In good correlation, AP-2 α -induced caspase activation also coincided with PARP cleavage (Fig. 1C). We also found that caspase activation is essential for AP-2 α -induced apoptosis as the pan caspase inhibitor, z-VAD-fmk, inhibited apoptotic cell death in Ad-AP2-infected cells (Fig. 2A). In addition, z-VAD-fmk treatment completely reversed the suppression of colony formation by AP-2 α (Fig. 2B; compare pCEP4/AP-2 α + z-VAD-fmk with pCEP4/AP-2 α).

Because both caspases 8 and 9 were activated in Ad-AP2-infected cells, we next determined the relative roles of the extrinsic (caspase 8-mediated) and intrinsic (caspase 9-mediated) pathways in AP-2 α -induced apoptosis. To address this point, we used caspase 8- and 9-specific inhibitors as well as an siRNA approach. The caspase 9-specific inhibitor (z-LEHD-fmk), but not caspase 8-specific inhibitor (z-IETD-fmk), efficiently inhibited AP-2 α -induced apoptosis as seen by the reduced percentage of cells with less than a 2N content of DNA (Fig. 2A). In good correlation, colony suppression by AP-2 α is completely reversed by z-LEHD-fmk but not by z-IETD-fmk (Fig. 2B). These results

suggest the primary utilization of caspase 9, but not caspase 8, by AP-2 α to induce apoptosis. To confirm this fact, we analyzed the role of FADD (the adapter protein, which acts upstream of caspase 8) and Apaf1 (which normally complexes with cytochrome *c* and caspase 9 to form an active apoptosome complex) in AP-2 α -induced apoptosis. We found that AP-2 α is able to induce apoptosis efficiently in cells where the expression of FADD is silenced by siRNA (see supplemental Fig. S1). On the other hand, AP-2 α failed to induce apoptosis in Apaf1-silenced cells (see below). Transfection of Apaf1-specific siRNA reduced the levels of Apaf1 transcript but not lamin transcript (Fig. 2C, lane 3) and Apaf1 protein (Fig. 2D, compare lane 4 with lane 3). Similarly, lamin siRNA reduced the lamin but not Apaf1 transcript (Fig. 2C, lane 2) and Apaf1 protein (Fig. 2D, lane 3). Neither siRNA inhibited the level of GAPDH transcript (Fig. 2C) and actin protein (Fig. 2D). Although Ad-AP2 induced apoptosis in the cells transfected with lamin-specific siRNA efficiently, it failed to do so in the cells transfected with Apaf1-specific siRNA (Fig. 2E). The percentage of apoptotic cells in Ad-AP2 virus-infected cells was reduced from 49.48% in the lamin siRNA-transfected group to 6.23% apoptotic cells in the AP-2 α siRNA-transfected group at 72 h after virus infection. These results suggest that caspase 8-mediated extrinsic pathway is dispensable, whereas caspase 9-dependent intrinsic pathway is required in AP-2 α -induced apoptosis.

Essential Role of Mitochondria in AP-2 α -induced Apoptosis—To understand better the importance of the intrinsic pathway of apoptosis, we investigated the role of mitochondria in AP-2 α -induced apoptosis by measuring mitochondrial membrane potential ($\Delta\psi_m$) as a measure of mitochondrial outer membrane permeabilization (MOMP) by using the potentiometric dye JC-1 (33) in Ad-AP2-infected cells. JC1 is a fluorescent cationic dye that aggregates in mitochondria in healthy cells and fluoresces red, whereas in apoptotic cells, wherein the mitochondrial membrane potential is reduced, it is diffused throughout the cells and assumes a monomeric form that fluoresces green. Because Ad-AP2 expresses green fluorescent protein also, which would interfere with JC1-based measurement of mitochondrial membrane potential, we decided to overexpress AP-2 α in the tetracycline-based (Tet-off) conditional expression system. AP-2 α expressed from the tetracycline-responsive promoter in stable SW480 cell clones was induced by infecting them with an adenovirus expressing tetracycline responsive transcriptional activator (Ad-tTA) in the absence of tetracycline (26). Overexpression of AP-2 α in SW480 cells resulted in 2.5-fold reduction in $\Delta\psi_m$ in comparison to control cells (Fig. 3A, compare bar 4 with bar 3). As expected, treatment of SW80 cells with valinomycin, which was used as a positive control because it induces apoptosis by disrupting $\Delta\psi_m$, resulted in 2.5-fold reduction in $\Delta\psi_m$ (Fig. 3A compare bar 2 with bar 1).

MOMP results in release of soluble mitochondrial intramembrane proteins such as cytochrome *c* and the IAP antagonist Smac/DIABLO (35, 36). To monitor the release of mitochondrial proteins into cytosol in AP-2 α overexpressing cells, we separated cytosolic and mitochondrial extracts from Ad-LacZ- and Ad-AP2-infected cells by biochemical fractionation and monitored the release of cytochrome *c* and Smac/DIABLO into cytosol. AP-2 α overexpression resulted in release of cytochrome *c* and Smac/DIABLO into cytoplasm (Fig. 3B) as against control cells infected with Ad-LacZ. The quality of fractionation was verified by monitoring the specific subcellular markers (β -actin as a cytoplasm-specific marker and oxidative complex 1 protein as a mitochondria-specific marker).

Next we sought to find out the importance of cytochrome *c* and Smac/DIABLO release into cytosol for AP-2 α -induced apoptosis. Because our results (see above) suggest that Apaf1 and caspase 9, which requires complex formation with cytosolic cytochrome *c* for their function, are essen-

AP-2 α Represses Bcl-2 to Induce Apoptosis

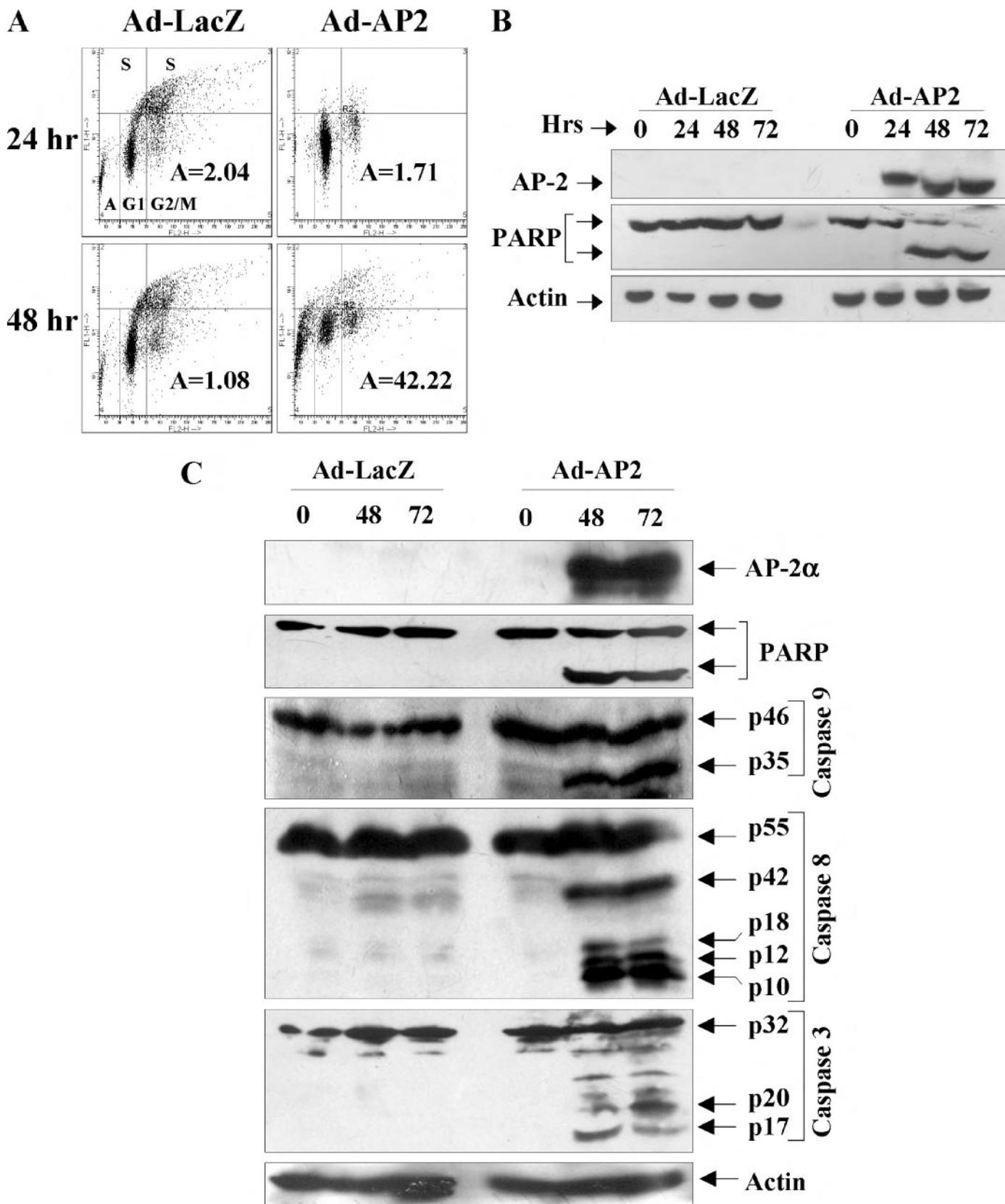


FIGURE 1. AP-2 α overexpression is cytotoxic by inducing apoptosis. *A*, H460 cells infected with either Ad-LacZ or Ad-AP2 virus at a 20 m.o.i. At the indicated time points, cells were harvested and subjected to double color flow analysis as described under "Experimental Procedures." The percentage of cells in the various phases of the cell cycle was calculated and is shown below. A indicates the percentage of cells containing less than a 2N amount of DNA (apoptotic cells), S indicates the cells undergoing DNA synthesis (S phase), G₁ indicates cells containing a 2N amount of DNA, and G₂/M indicates cells containing a 4N amount of DNA. *B*, H460 cells infected with either Ad-LacZ or Ad-AP2 virus at a 20 m.o.i. At the indicated time points, cells were harvested and subjected to Western blot analysis for AP-2 α , PARP, and actin as described under "Experimental Procedures." *C*, H460 cells infected with either Ad-LacZ or Ad-AP2 virus at a 20 m.o.i. At the indicated time point, cells were harvested and subjected to Western blot analysis for AP-2 α , PARP, caspase 8, caspase 9, caspase 3, and actin proteins.

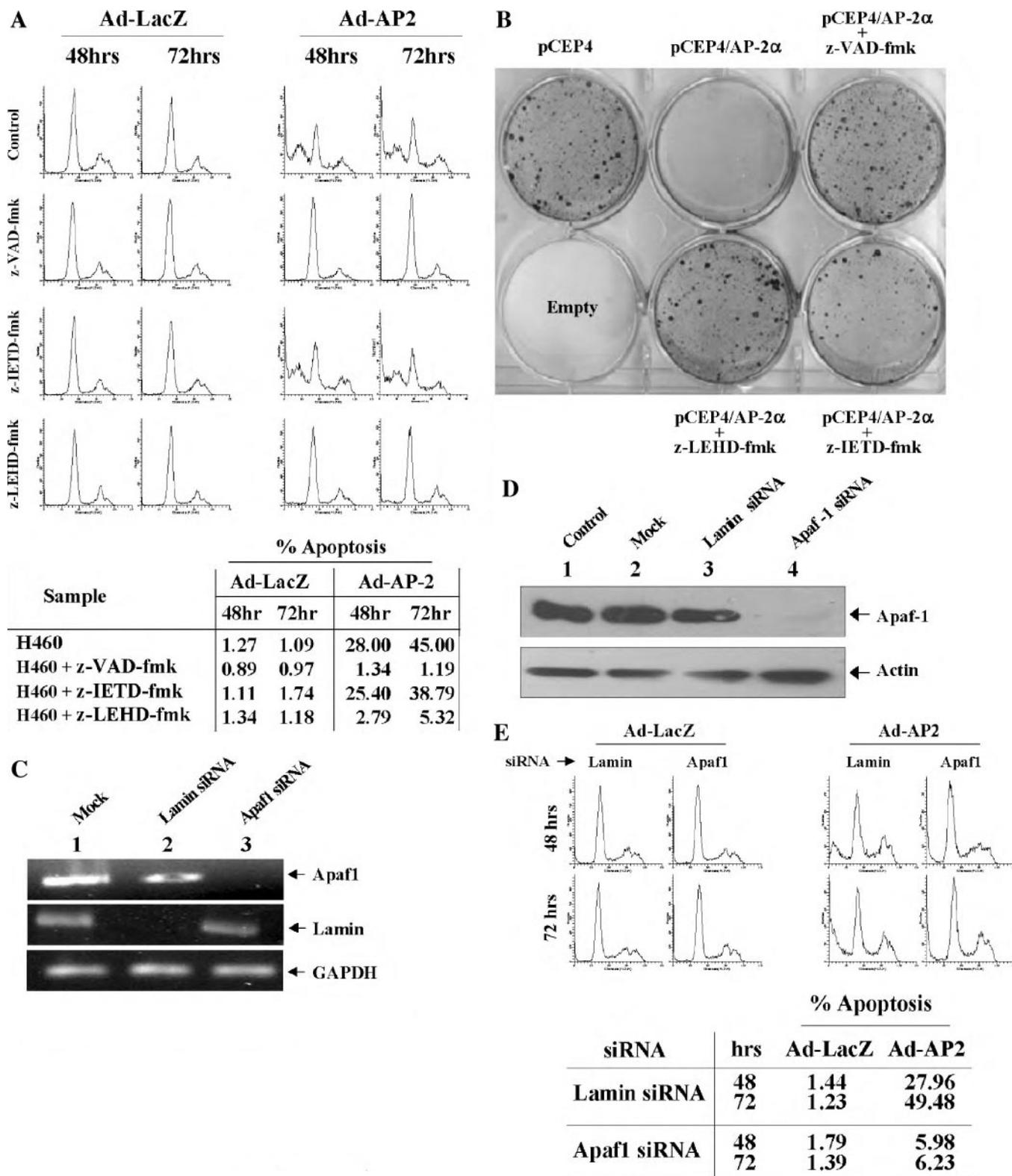


FIGURE 2. AP-2 α induces apoptosis through caspase 9-dependent mitochondrial pathway. *A*, H460 cells were either infected with either Ad-LacZ or Ad-AP2 at a 20 m.o.i. in the absence or presence of various inhibitors as indicated. Cells were harvested at the indicated time points and subjected for FACS analysis. Apoptotic population measured as cells with less than 2N DNA content has been quantitated and presented in the table. *B*, SW480 cells were either transfected with pCEP4 or pCEP4/AP2 α . After 48 h of transfection, cells were either left untreated or treated with z-VAD-fmk (25 μ M), z-LEHD-fmk (40 μ M), or z-IETD-fmk (40 μ M), and all the transfected samples were selected for hygromycin resistance. Medium was removed every 4th day and replenished with respective inhibitors and hygromycin (250 μ g/ml). After the appearance of visible colonies, the plates were then stained with Coomassie Blue (CBB R-250) and shown. *C*, SW480 cells were mock-transfected or transfected with lamin or Apaf1 siRNA. After 4 days of siRNA transfection, total RNA was prepared and subjected to RT-PCR analysis for Apaf1, lamin, and GAPDH transcripts. *D*, SW480 cells were not transfected (Control) or were mock-transfected or transfected with lamin or Apaf1 siRNA. After 4 days of siRNA transfection, total cell lysate was prepared and subjected to Western blot analysis for Apaf1 and Actin proteins. *E*, SW480 cells were transfected with lamin or Apaf1 siRNA. After 48 h of transfection, the cells were infected with Ad-LacZ or Ad-AP2 at a 20 m.o.i. Cells were harvested at the indicated time points post-infection and subjected to FACS analysis. Apoptotic population measured as cells with less than a 2N content of DNA is quantitated and shown at the right.

AP-2 α Represses Bcl-2 to Induce Apoptosis

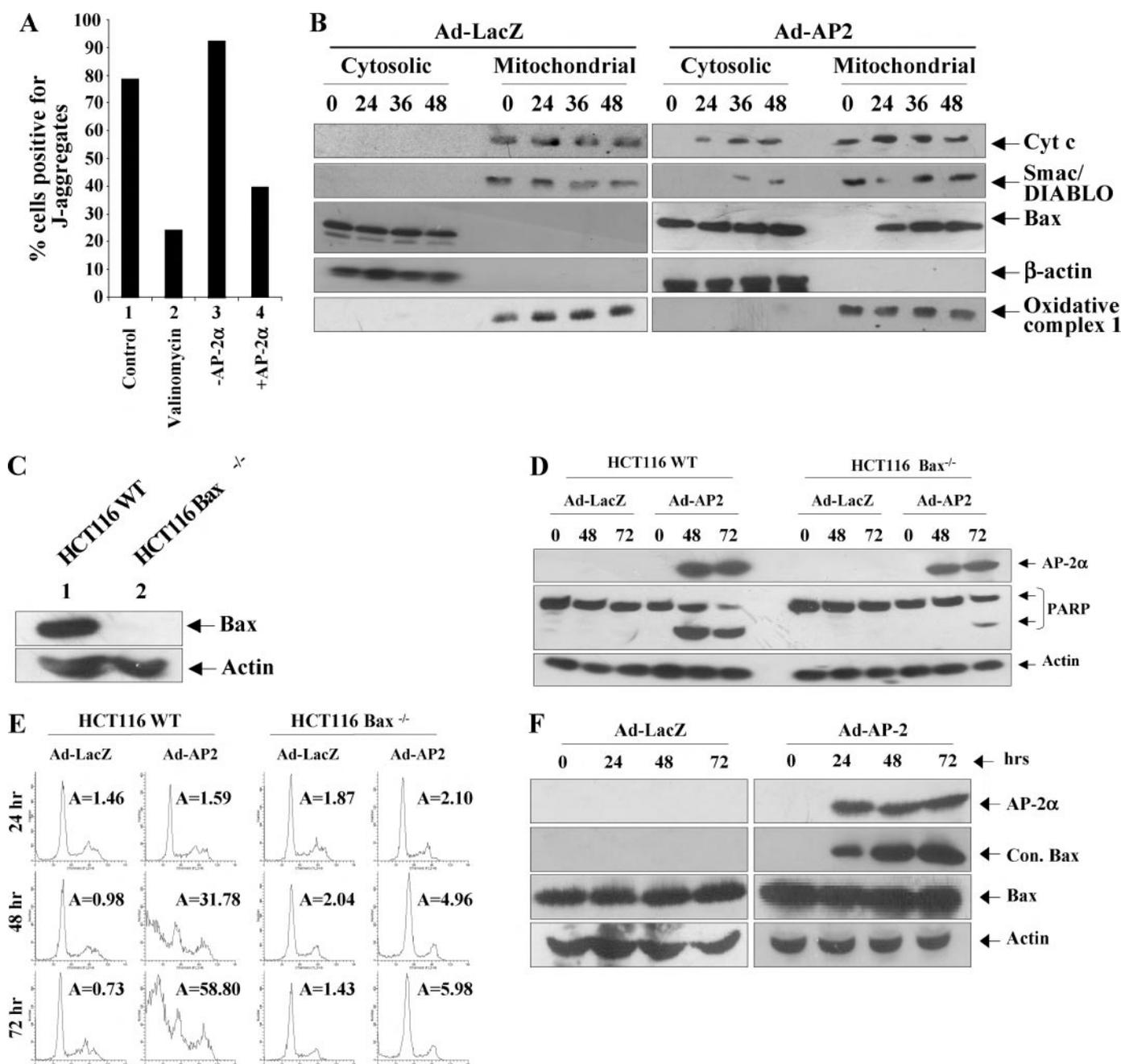


FIGURE 3. Role of mitochondria and Bax in AP-2 α -induced apoptosis. *A*, mitochondrial membrane potential is measured using potentiometric dye JC-1. Valinomycin, a mitochondrial membrane potential uncoupler, is used as a positive control. Tetracycline-inducible clones (Tet-off) were grown in the presence (uninduced) or absence (induced) of tetracycline (1 μ g/ml) as described before (26). After 48 h of AP-2 α induction, cells were harvested and subjected to flow cytometry analysis as described in under "Experimental Procedures." *B*, mitochondrial and cytosolic fractions of Ad-LacZ- or Ad-AP2-infected cells were isolated at the indicated time points and subjected to Western blot analysis for cytochrome *c* (Cyt *c*), Smac/DIABLO, Bax, and β -actin, NADH-ubiquinone oxidoreductase (Oxidative Complex I). *C*, Western blot analysis of HCT116 WT and HCT116 Bax^{-/-} for the Bax and actin proteins. *D*, HCT116 WT or HCT116 Bax^{-/-} cells were infected with Ad-LacZ or Ad-AP2 at a 20 m.o.i. Cells were harvested at the indicated time points and subjected to Western blot analysis for AP-2 α , PARP, and actin. *E*, HCT116 WT and HCT116 Bax^{-/-} cells were either left uninfected or infected with either Ad-LacZ or Ad-AP2 at a 20 m.o.i. Cells were harvested at the indicated time points and subjected for FACS analysis. The apoptotic population (A) was quantitated and shown. *F*, HCT116 WT cells infected with either Ad-LacZ or Ad-AP2 at a 20 m.o.i. Cells were harvested at the indicated time points and subjected to immunoprecipitation for Bax protein using conformation-specific antibody 6A7, which recognizes only conformationally changed active Bax (Con.Bax). Immunoprecipitated Bax was detected by Western blotting using polyclonal antibody for Bax. Total protein lysate of the same samples prepared at different time points was subjected to Western blot analysis to see the AP-2 α , Bax, and actin expression.

tial for AP-2 α -induced apoptosis, we conclude that cytochrome *c* release is essential for AP-2 α -induced apoptosis. However, we found that the IAP inhibitor Smac/DIABLO may have a very limited role in AP-2 α -induced apoptosis (see below). The apoptosis induction by AP-2 α in cells where Smac/DIABLO is silenced by siRNA was reduced by 33–45% (see supplemental Fig. S2). In addition, we found that AP-2 α down-regulated the expression of IAP protein Survivin, thus making

Smac/DIABLO dispensable for AP-2 α -induced apoptosis (see supplemental Fig. S2). Thus, these results suggest that mitochondria play a major role in AP-2 α -induced apoptosis.

Role of Bax in Apoptosis Induction by AP-2 α —Because MOMP is mainly mediated and controlled by Bcl-2 family members (36), we then analyzed the role of pro-apoptotic Bcl-2 family member Bax. First we determined whether AP-2 α -induced MOMP is accompanied by Bax relo-

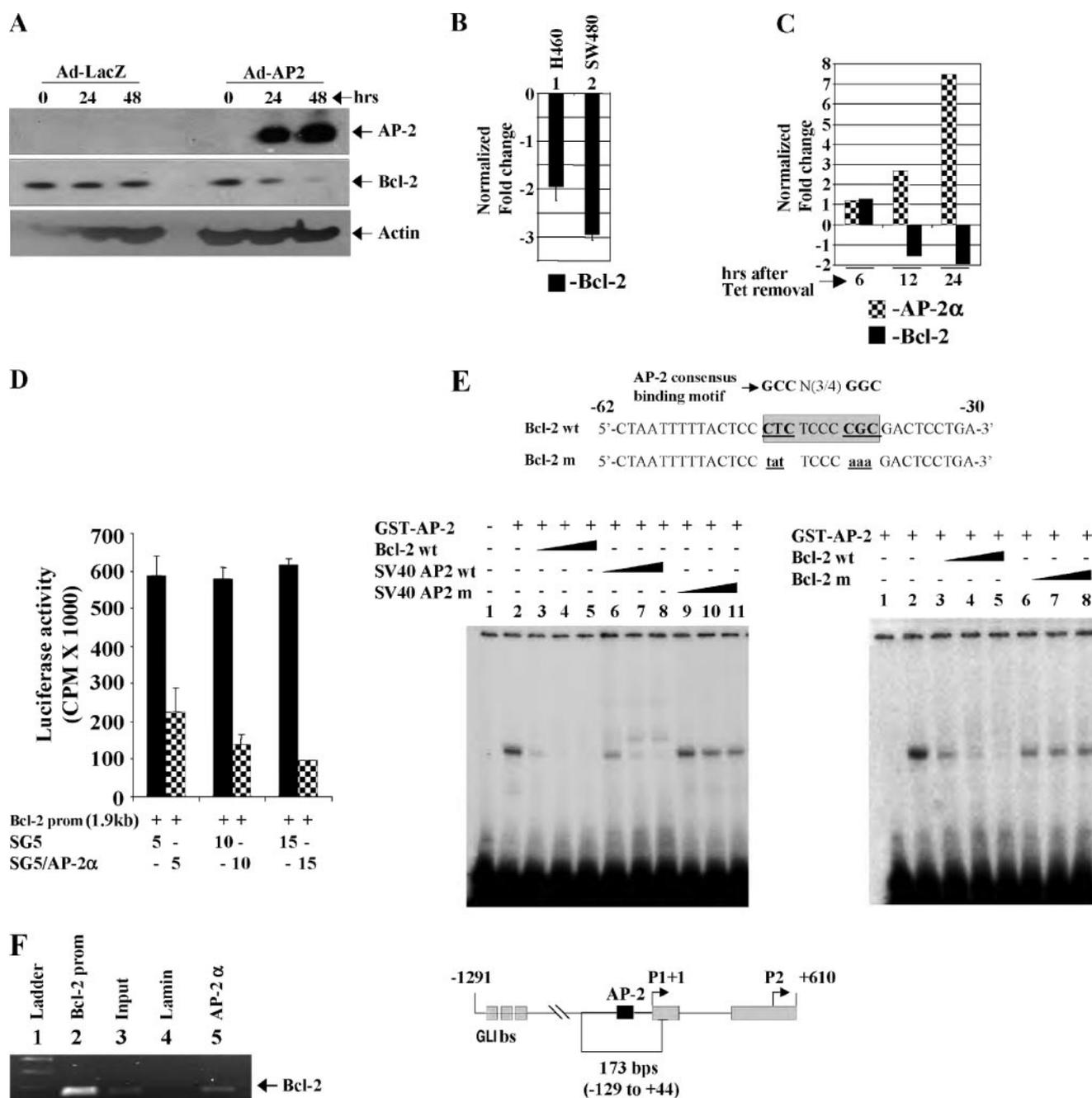


FIGURE 4. AP-2 α induces apoptosis by repressing Bcl-2. *A*, SW480 cells were infected with Ad-LacZ or Ad-AP2 at a 20 m.o.i. Cells were harvested at the indicated time points and subjected to Western blot analysis for AP-2 α , Bcl-2 and Actin proteins. *B*, SW480 and H460 cells infected with either Ad-LacZ or Ad-AP2 (20 m.o.i.). Total RNA was isolated after 24 h of infection and subjected to quantitative real time RT-PCR analysis for AP-2 α , Bcl-2, and GAPDH transcripts. The -fold change for each gene was calculated as the ratio between specific transcript levels in the Ad-AP2- and Ad-LacZ-infected samples and was normalized to the levels of GAPDH transcript. The normalized -fold change for AP-2 α in H460 and SW480 cells was found to be 38.80 and 12.13, respectively (data not shown). *C*, total RNA was made from the Tet-Off AP-2 α clone at the indicated time points in the presence (uninduced) or absence (induced) of tetracycline (1 μ g/ml) and subjected to quantitative real-time RT-PCR analysis for AP-2 α , Bcl-2, and GAPDH transcripts. The -fold change for each gene was calculated as the ratio between specific transcript levels in the -tet and +tet conditions for each time points and was normalized to the levels of GAPDH transcript. *D*, SW480 colon cancer cells were transfected with 5 μ g of Bcl-2prom (1.9 kilobases) (indicated by +) and pSG5 vector or pSG5/AP-2 α expression plasmid at indicated amounts. Transfections were carried out by using Escort III (sigma). Lysates were prepared and analyzed for luciferase reporter activity 24 h post-transfection as described in under "Experimental Procedures." pUC18 plasmid DNA was added to keep the total amount of DNA constant per transfection. 1 μ g of the plasmid expressing β -galactosidase was transfected in each well and was further used as an internal control for normalization of transfection efficiency variations. *E*, electrophoretic mobility shift assay was performed using purified GST-AP-2 DNA binding domain (amino acids 165–437) as described in under "Experimental Procedures." Competitions were performed by adding increasing amounts (0.2, 1, and 2 pmol) of specific (*Bcl-2 wt* and *SV40 AP2 wt*) or nonspecific (*SV40 AP2 m*) competitor as indicated (*left panel*). On the *right* competition was carried out by adding increasing amounts (0.2, 1, and 2 pmol) of specific (*Bcl-2 wt*) or nonspecific (*Bcl-2 m*) competitor. *F*, chromatin from cross-linked subconfluent Ad-AP2-infected cells was immunoprecipitated with antibodies specific for AP-2 α (*lane 5*) or nonspecific antibody lamin B (*lane 4*). *bcl-2* sequence was detected by PCR analysis of eluted DNA. A known amount of chromatin from each sample was removed before immunoprecipitation and used for PCR amplified (*Input*; *lane 3*). Plasmid DNA carrying Bcl-2 promoter (Bcl-2prom (1.9 kilobases)) was used as a positive control for PCR (*lane 2*). A schematic of the amplified region Bcl-2 promoter is shown on the *right*. *GLI*-bs, glioma-associated oncogene homolog 1 binding sequence.

cation into mitochondria from cytoplasm. Overexpression of AP-2 α by Ad-AP2 infection resulted in translocation of Bax into mitochondria as early as 24 h after virus infection, whereas in Ad-LacZ-infected cells Bax

remained in the cytoplasm (Fig. 3*B*). In addition, to find out the importance of Bax in AP-2 α -induced apoptosis, we analyzed the ability of AP-2 α to induce apoptosis in HCT116 *bax*^{-/-}, which is a somatic

transcriptionally regulate Bcl-2 family members to induce apoptosis, we determined the levels of various Bcl-2 family members in AP-2 α overexpressing cells. Because our results suggest that Bax is essential for AP-2 α -induced apoptosis, we first examined the levels of Bax in Ad-AP2-infected cells. We found no change in Bax transcript levels (data not shown) and Bax protein levels (Fig. 3F) between Ad-LacZ- and Ad-AP2-infected cells. It has been shown that translocation of Bax into the outer mitochondrial membrane involves a conformational change that exposes the N terminus and the hydrophobic C terminus that targets mitochondria (38). To examine the possibility that Bax might be activated by conformational change in AP-2 α overexpressing cells, we performed immunoprecipitation experiments with anti-Bax 6A7 antibody that recognizes only the conformationally changed Bax protein. As seen in Fig. 3F, Bax underwent a conformational change at 24 h after Ad-AP2 virus infection, which further increased in a time-dependent fashion. Bax conformational change in AP-2 α overexpressing cells also correlated well with the release of cytochrome *c* into cytosol, which occurs by 24 h, and it precedes apoptosis induction, which occurs at 48 h after virus infection as seen by the appearance of apoptotic cells documented by flow cytometry, PARP cleavage, and caspase 3, 8, and 9 activation (Fig. 1, A–C). Thus, these results indicate that, although Bax protein level does not change, Bax undergoes a conformational change to form an active form in AP-2 α -overexpressing cells, thus playing a major role in AP-2 α -induced apoptosis.

We then focused our attention on other members of Bcl-2 family belonging to both anti-apoptotic and BH3-only groups. Although most of them remain unaltered in their transcript levels in AP-2 α overexpressing cells, we found that the level of Bcl-2 protein, the prototype anti-apoptotic member of Bcl-2 family, is reduced drastically in a time-dependent fashion in SW480 cells overexpressing AP-2 α (Fig. 4A). We also found that Bcl-2 transcript level is decreased in H460 and SW480 cells upon Ad-AP2 virus infection by 1.95- and 2.96-fold, respectively (Fig. 4B). In addition, upon overexpression of AP-2 α in a tet-off-inducible system, Bcl-2 transcripts decreased 1.59-fold by 12 h, which further decreased to 1.95-fold by 24 h after tetracycline removal (Fig. 4C). The time-dependent decrease in Bcl-2 transcript levels also correlated well with the increasing levels of AP-2 α transcripts after tetracycline removal (Fig. 4C). The transcriptional repression of Bcl-2 by AP-2 α appears to be due decreased promoter activity as exogenous expression of AP-2 α decreased the Bcl-2 promoter activity in a dose-dependent manner (Fig. 4D).

Next, to determine whether transcriptional repression of Bcl-2 by AP-2 α is due to a direct effect, we analyzed the Bcl-2 promoter for the presence of AP-2 binding sequence. We found an AP-2 binding motif 5'-CTCTCCCCGC-3' at -48 to -39 of Bcl-2 promoter. We carried out electrophoretic mobility shift assay with ³²P-end-labeled -62 to -39 Bcl-2 probe and bacterially expressed GST-AP-2 α fusion. The AP-2 binding sequence located between -62 to -39 of Bcl-2 promoter bound to GST-AP-2 α (Fig. 4E, left panel, lane 2) and is specific as it is competed by unlabeled Bcl-2 oligonucleotide as well as by oligonucleotides containing the AP-2 binding site of the SV40 enhancer but not by oligonucleotides containing mutated SV40 AP-2 binding site (Fig. 4E, left panel, compare lanes 3–5, 6–8, and 9–11 with lane 2, respectively). The specificity of AP-2 α binding to -48 to -39 Bcl-2 promoter sequence is also confirmed by the fact that the unlabeled Bcl-2 oligo carrying mutated AP-2 binding motif (5'-CTCTCCCCGC-3' is mutated to 5'-TATTCCCAA-3') failed to compete efficiently (Fig. 4E, right panel, compare lanes 6–8 with 3–5). In addition, to find out whether AP-2 α binds Bcl-2 promoter *in vivo*, we analyzed a 173-bp fragment that spans -129 to +44 carrying the AP-2 binding sequence

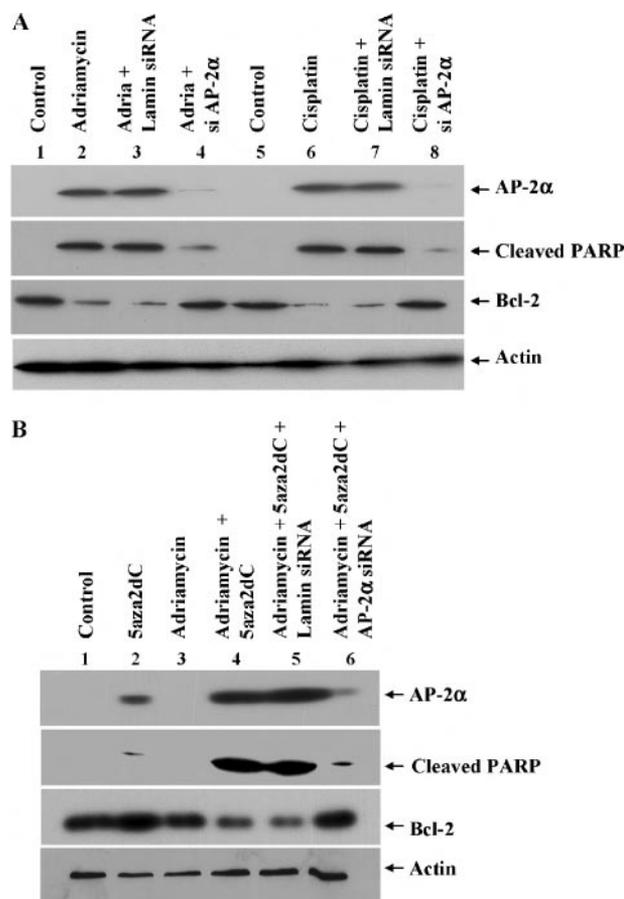


FIGURE 6. Chemotherapy-induced endogenous AP-2 α down-regulates Bcl-2 and induces apoptosis. A, SW480 cells were left mock-transfected (lanes 1, 2, 5, and 6) or transfected with the mentioned siRNAs. After 48 h of transfection cells were treated with adriamycin (0.8 μ g/ml) or cisplatin (4 μ g/ml) for 48 h, and then the cells were harvested and subjected to Western blot analysis for AP-2 α , cleaved PARP, Bcl-2, and actin proteins. B, MDA-MB-231 cells were left mock-transfected (lanes 1–4) or transfected with the mentioned siRNAs. After 48 h of transfection cells were treated by 5Aza2dC (5 μ M) for 36 h and followed by treatment with adriamycin (0.8 μ g/ml) for 48 h. Cells were harvested then and subjected to Western blot analysis for AP-2 α , cleaved PARP, Bcl-2, and actin proteins.

(Fig. 4F) by chromatin immunoprecipitation assay. In SW480 cells infected with Ad-AP2, AP-2 α bound to this fragment efficiently and is specific as a nonspecific antibody (lamin) failed to bind (Fig. 4G, compare lanes 5 and 4). These results conclusively prove that AP-2 α transcriptionally represses Bcl-2 by binding directly to its promoter.

To find out whether Bcl-2 down-regulation is necessary for AP-2 α -induced apoptosis, we determined whether ectopic expression of Bcl-2 could block cytotoxicity and apoptosis induced by AP-2 α . First, we checked the effect of Bcl-2 on the ability of AP-2 α to suppress the colony formation. Although AP-2 α expression vector alone efficiently inhibited colony formation, it failed to inhibit colony formation when cotransfected along with Bcl-2 expression vector (Fig. 5A, compare bar 3 with bar 4). As expected, Bcl-2 expression vector alone did not inhibit colony formation (Fig. 5A, bar 2). We then tested the ability of Ad-AP2 to induce apoptosis in Bcl-2 stable cell lines derived from SW480 cells. Two independent Bcl-2 stable clones of SW480 (#3 and #15) expressed increased amounts of Bcl-2 in comparison to a vector stable clone (SW480/Neo) (Fig. 5B, compare lanes 2 and 3 with lane 1). AP-2 α failed to induce apoptosis efficiently in the Bcl-2 stable cell line as seen by reduced PARP cleavage and reduced apoptotic cells (Fig. 5, C and D). Although Ad-AP2-infected SW480/Neo cells showed efficient PARP cleavage by 48 h, SW480/Bcl-2 #3 clone, upon Ad-AP2 infection, showed no PARP cleavage by 48 h and very minimal PARP cleavage by

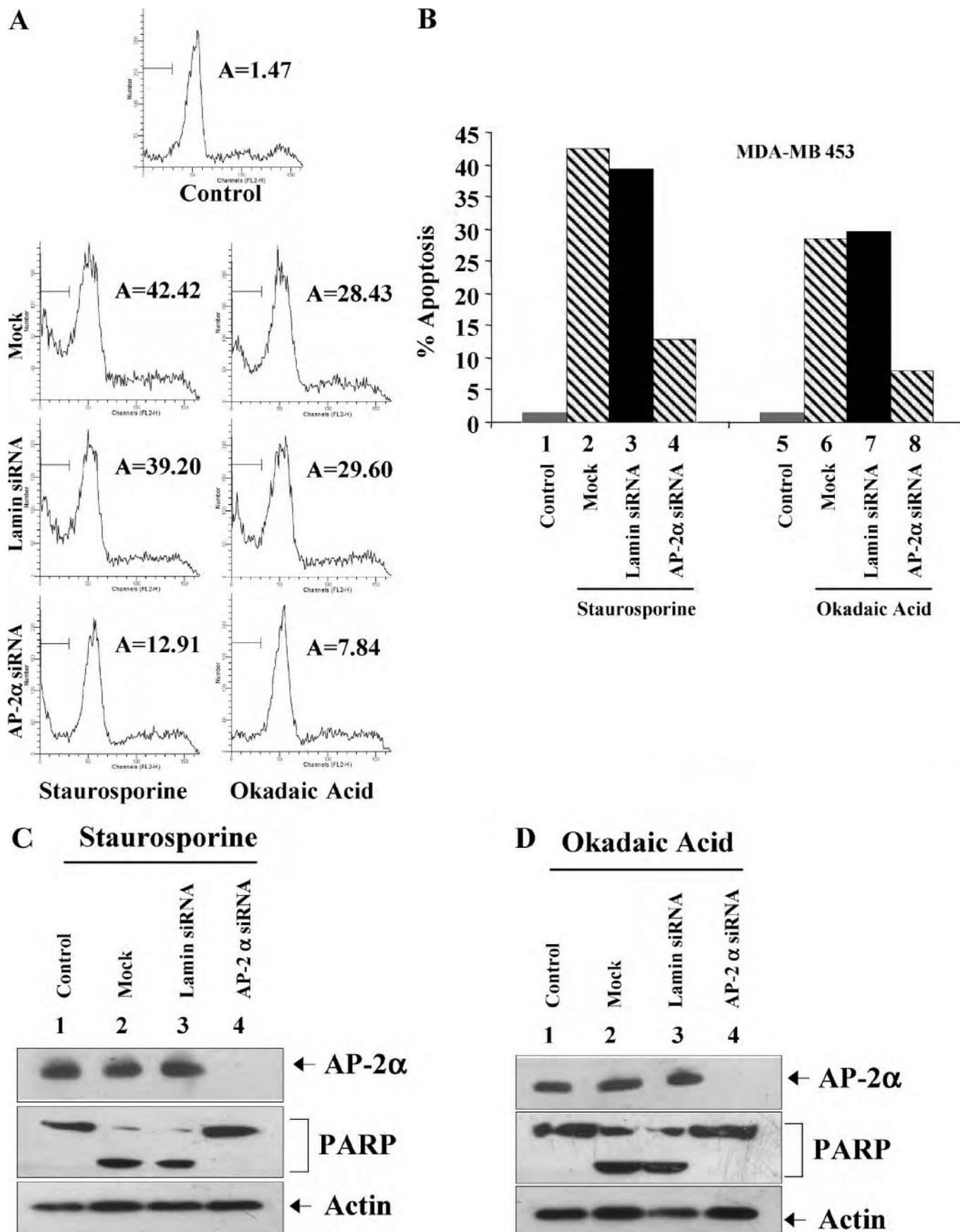


FIGURE 7. **Staurosporine- and okadaic acid-induced apoptosis is reversed by AP-2 α siRNA.** *A*, MDA-MB-453 cells were left untransfected (*Control*) or mock-transfected or transfected with lamin siRNA or AP-2 α siRNA. 48 h post-transfection cells were treated with staurosporine or okadaic acid for the next 48 h. At the end of the drug treatment cells were harvested and subjected to single color FACS analysis as described under "Experimental Procedures." *B*, % apoptosis as measured cells with less than a 2N content of DNA from *A*

72 h (Fig. 5C). Similarly, Ad-AP2 infection resulted in a severalfold increase in apoptotic cells in SW40/Neo cells in a time-dependent manner (Fig. 5D; 0.78% at 24 h is increased to 24.89% by 48 h and 42.91% by 72 h). However, Ad-AP2 infection failed to induce any significant apoptosis in SW480/Bcl-2 #3 clone (Fig. 5D; 2.10% at 24 h is increased to 5.73% by 48 h and 6.54% by 72 h). As expected in both cell lines, Ad-LacZ infection did not result in apoptosis induction (Fig. 5D). Taken together, we conclude that down-regulation of Bcl-2 is essential for AP-2 α -induced apoptosis.

Chemotherapy-induced Endogenous AP-2 α Down-regulates Bcl-2 and Induces Apoptosis—We have previously shown that AP-2 α is an important determinant of cancer cell chemosensitivity (26). Both chemotherapy-induced endogenous AP-2 α and methylation inhibitor (5aza2dC)-induced re-expression of endogenous AP-2 α modulated the chemosensitivity. To demonstrate the physiological relevance of apoptosis induction by AP-2 α , we analyzed apoptosis induction by AP-2 α upon chemotherapy treatment or 5aza2dC treatment. Upon treatment of SW480 cells with adriamycin or cisplatin resulted in increase in endogenous AP-2 α (Fig. 6A, compare lane 2 with lane 1 and lane 6 with lane 5). Increased endogenous AP-2 α by chemotherapy resulted in down-regulation of Bcl-2 and induction of apoptosis, as seen by the appearance of cleaved PARP (Fig. 6A, compare lane 2 with lane 1 and lane 6 with lane 5). Both apoptosis induction and Bcl-2 down-regulation in the above experiment is AP-2 α -specific as cells transfected with AP-2 α siRNA, but not lamin siRNA, failed to increase AP-2 α levels, induce apoptosis, and down-regulate Bcl-2 (Fig. 6A, compare lane 4 with lane 3 and lane 8 with lane 7). In addition, we found similar results in MDA-MB-231 cells where AP-2 α is re-expressed by methylation inhibitor. 5aza2dC treatment resulted in re-expression of AP-2 α in MDA-MB-231 cells (Fig. 7B, lane 2 with lane 1). The addition of adriamycin to 5aza2dC-treated MDA-MB-231 cells resulted in a further increase in AP-2 α , which correlated with down-regulation of Bcl-2 and induction of apoptosis, as seen by increased PARP cleavage (Fig. 6B, lane 4 with lane 2), and is AP-2 α -specific because similar changes are abolished by transfection of cells with AP-2 α siRNA, but not by lamin siRNA (Fig. 6B, compare lane 6 with lane 5).

Okadaic Acid or Staurosporine Treatment Induces AP-2 α -dependent Apoptosis in Breast Cancer Cells—An inefficient proteosomal degradation pathway has been found to be responsible for high levels of AP-2 α in certain breast cancer-derived cells (39). We hypothesized that the growth inhibitory functions of AP-2 α is inhibited in these cells by survival signaling, inhibition of which might restore its functions. We used the breast cancer cell line MDA-MB-453 cell line, which carries elevated levels of AP-2 α (Fig. 7, C and D, lane 1) as previously reported (39). We found that treatment of MDA-MB-453 cells with staurosporine or okadaic acid resulted in apoptosis induction, as evidenced by an increase in the percentage of cells with less than 2N content of DNA (Fig. 6, A and B, compare lane 2 with lane 1) and increased PARP cleavage (Fig. 7C, compare lane 2 with lane 1, and D, compare lane 2 with lane 1). The increase in apoptosis is mainly dependent on the presence of AP-2 α as simultaneous silencing of AP-2 α expression by transfection of cells with specific siRNA resulted in drastic reduction of apoptosis (Fig. 7, A and B, compare lane 4 with lane 3 and compare lane 8 with lane 7; C, compare lane 4 with lane 3; D, compare lane 4 with lane 3). These results suggest that AP-2 α can be activated in breast cancer cells to induce apoptosis by inhibiting survival signaling, which is sensitive to staurosporine and okadaic acid.

DISCUSSION

In this study we demonstrate that AP-2 α -induced apoptosis utilizes mainly the mitochondrial pathway of apoptosis, which is demonstrated by the following experiments. We found that the addition of caspase 9 inhibitor or siRNA silencing of Apaf1 completely abrogated the apoptosis induction by AP-2 α , whereas the addition of caspase 8-specific inhibitor z-IETD-fmk or siRNA silencing of the adapter protein FADD did not have any significant effect on AP-2 α -induced apoptosis. In addition, AP-2 α -overexpressing cells showed MOMP, which is accompanied by reduction in mitochondrial membrane potential ($\Delta\psi_m$), release of cytochrome *c* and Smac/DIABLO into cytosol, and translocation of Bax into mitochondria. Although cytochrome *c* is found to be essential, Smac/DIABLO may play a limited role in AP-2 α -mediated apoptosis. Moreover, we found AP-2 α failed to induce apoptosis in cells lacking Bax. Although the total Bax levels remain unchanged in AP-2 α -overexpressing cells, we found the presence of conformationally changed active form of Bax. We also found that AP-2 α binds to the Bcl-2 promoter, specifically leading to transcriptional repression of Bcl-2, and is essential for AP-2 α -induced apoptosis as ectopic expression of Bcl-2 blocked the same. Finally, we show that in cancer cells overexpressing non-functional AP-2 α , apoptosis could be induced by blocking okadaic acid- or staurosporine-sensitive survival signaling pathways in an AP-2 α -dependent manner.

The apoptotic signaling pathway that leads to caspase activation can be subdivided into two major categories: death receptor-mediated and mitochondria-mediated pathways (40, 41). We provide multiple evidence for the intrinsic pathway along with mitochondria that play an important role in AP-2 α -induced apoptosis. Neither caspase 8 inhibitor nor siRNA silencing of death receptor adaptor protein, FADD, significantly affected AP-2 α -induced apoptosis. On the contrary, caspase 9 inhibitor completely inhibited the apoptosis induced by AP-2 α . In addition, AP-2 α adenovirus-infected cells showed a reduction in mitochondrial membrane potential, which is suggestive of permeabilization of the mitochondrial outer membrane. This was also correlated with the release of mitochondrial proteins cytochrome *c* and Smac/DIABLO into cytosol in AP-2 α -overexpressing cells.

The permeabilization of the mitochondrial outer membrane leads to cell death because of the release of soluble mitochondrial intramembrane proteins such as cytochrome *c* and the disruption of essential mitochondrial functions (36). Upon release from mitochondria, cytochrome *c* binds to Apaf1, leading to the formation of apoptosome complex, which then recruits procaspase 9 leading to its activation through oligomerization (42). Apart from cytochrome *c*, another group of proteins released from mitochondria upon MOMP are IAP antagonists including Smac/DIABLO, HtrA2/Omi, and GSPT1/eRF3. Although IAPs act as a brake for apoptosis by inhibiting activated caspases, IAP antagonists bind IAPs to inhibit them in a competitive manner. Smac/DIABLO is also released along with the cytochrome *c* during apoptosis to neutralize the inhibitory activity of IAPs and promote cytochrome *c*-dependent caspase activation (35). Our results indicate that both cytochrome *c* and Smac/DIABLO are released from mitochondria in AP-2 α -overexpressing cells. However, cytochrome *c* release seems to be essential for AP-2 α -induced apoptosis as the siRNA-based silencing of its downstream target Apaf1 resulted in the complete abrogation of apoptosis in Ad-AP2-infected cells. Unlike Apaf1, silencing of Smac/DIABLO resulted in only partial reduction of apoptosis in AP-2 α -over-

is quantitated and shown. C, MDA-MB-453 cells were left untransfected (Control) or mock-transfected or transfected with lamin siRNA or AP-2 α siRNA. 48 h post-transfection cells were treated with staurosporine for next 48 h. At the end of the drug treatment, cells were harvested and subjected to Western blot analysis for AP-2 α , PARP, and actin proteins. D, MDA-MB-453 cells were left untransfected (Control) or mock-transfected or transfected with lamin siRNA or AP-2 α siRNA. 48 h post-transfection cells were treated with okadaic acid for the next 48 h. At the end of the drug treatment cells were harvested and subjected to Western blot analysis for AP-2 α , PARP, and actin proteins.

AP-2 α Represses Bcl-2 to Induce Apoptosis

expressing cells. This suggests the possibility that IAPs may have only a limited role in controlling the apoptosis induced by AP-2 α . Indeed, we found that the transcript levels of Survivin, a member of IAP family, is reduced severalfold in cells overexpressing AP-2 α . Antisense oligonucleotide-based inhibition of Survivin in H460 lung cancer cells has been shown to sensitize the cells to radiation (43). p53 has also been shown to transcriptionally down-regulate Survivin by binding to its promoter, which is believed to contribute to p53-dependent apoptosis (44). Therefore, we conclude that cytochrome *c* release is essential and IAP inhibitor is less essential as the IAP Survivin is down-regulated by AP-2 α for apoptosis induction by AP-2 α .

The Bcl-2 family of proteins plays a critical role in the mitochondrial pathway of apoptosis (36). The antiapoptotic Bcl-2 family proteins like Bcl-2 and Bcl-XL inhibit the release of certain pro-apoptotic factors from mitochondria, whereas the pro-apoptotic Bcl-2 family molecules (BH3 only and multidomain BH1–3 proteins) induce the release of mitochondrial apoptogenic molecules into the cytosol. Evidence suggests that the multidomain pro-apoptotic proteins Bax and Bak are absolutely required for apoptosis induction by diverse intrinsic death stimuli (45). Our results clearly demonstrate an important role for Bcl-2 family members in AP-2 α -induced apoptosis. Although the total Bax levels (both transcript and protein) did not change, Bax is found to be activated by conformational change leading to its translocation into mitochondria in Ad-AP2-infected cells. Moreover, we found that Bax is essential for apoptosis induction by AP-2 α . We found the levels of Bcl-2 transcript and protein reduced severalfold in AP-2 α -overexpressing cells. Furthermore, we show that Bcl-2 down-regulation by AP-2 α involves direct binding AP-2 α onto an AP-2 binding motif located between –48 to –39 of Bcl-2 promoter. We also provide evidence for the fact that Bcl-2 down-regulation by AP-2 α is necessary for it to induce apoptosis. Forced expression of Bcl-2 into cells, either from a transiently transfected cDNA or stably integrated copy of Bcl-2, resulted in complete abrogation of apoptosis induction by AP-2 α . Therefore, we propose that the mechanism of apoptosis induction by AP-2 α may involve transcriptional down-regulation of Bcl-2, which indirectly may promote Bax activation. In addition to this, repression of the IAP family member Survivin by AP-2 α may also play a role in AP-2 α -induced apoptosis. AP-2 α has been shown to negatively regulate the expression of several genes, some of which involves direct binding of AP-2 α to their promoter sequences like K3 keratin and vascular endothelial growth factor (19, 46). Thus, we conclude that AP-2 α induces apoptosis by down-regulating Bcl-2 and utilizes mitochondrial pathway of apoptosis induction.

We also provide evidence for physiological relevance of apoptosis induction by AP-2 α . Chemotherapy-induced endogenous AP-2 α in SW480 cells resulted in Bcl-2 down-regulation and apoptosis induction in an AP-2 α -dependent manner. Similarly, chemotherapy-mediated increase of re-expressed AP-2 α in 5aza2dC-treated MDA-MB-231 cells resulted in down-regulation of Bcl-2 and apoptosis induction in an AP-2 α -dependent manner.

Although the endogenous level of AP-2 α in most cell lines has been found to be low, increased levels have been reported in certain breast cancer cells (39). Because AP-2 α is a potent growth inhibitory protein, the high levels seen in these cells might reflect a functionally inactive state. Adenovirus oncogene E1A has been shown to stabilize the tumor suppressor protein p53 while simultaneously inhibiting its transcriptional activation function (47). Similarly, regulation of AP-2 α at post-transcriptional level has been reported previously. AP-2 α activity has been shown to be stimulated by 12-*O*-tetradecanoylphorbol-13-acetate or okadaic acid treatment via a posttranscriptional mechanism (48, 49). Similarly, protein kinase A-induced phosphorylation of AP-2 α has been

shown to increase the ability of AP-2 α to transactivate apolipoprotein E (*APOE*) promoter (50). Our results show that treatment of MDA-MB-453 cells with okadaic acid or staurosporine resulted in AP-2 α -dependent apoptosis (Fig. 6). Protein phosphatase 2A, which is inhibited by okadaic acid, is known to regulate many kinases particularly involved in survival signaling. For example, protein phosphatase 2A regulates Akt/protein kinases B and C and mTOR, all of which promote cell survival (51–53). Similarly, protein kinase C, which is inhibited by staurosporine, has been implicated in neoplastic transformation (54), regulation of apoptosis (55), and multidrug-resistant phenotype (56). Because our results show that okadaic acid and staurosporine induce AP-2 α -dependent apoptosis, it is possible that protein phosphatase 2A- and protein kinase C-regulated growth promoting, anti-apoptotic, and tumorigenic signaling pathways could lead to AP-2 α functional inactivation in AP-2 α -overexpressing cancer cells. Further studies are in progress to dissect the pathways that leads to inactivation of AP-2 α .

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REFERENCES

1. Mitchell, P. J., Wang, C., and Tjian, R. (1987) *Cell* **50**, 847–861
2. Nottoli, T., Hagopian-Donaldson, S., Zhang, J., Perkins, A., and Williams, T. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 13714–13719
3. Schorle, H., Meier, P., Buchert, M., Jaenisch, R., and Mitchell, P. J. (1996) *Nature* **381**, 235–238
4. Garcia, M. A., Campillos, M., Ogueta, S., Valdivieso, F., and Vijquez, J. (2000) *J. Mol. Biol.* **301**, 807–816
5. Mohibullah, N., Donner, A., Ippolito, J. A., and Williams, T. (1999) *Nucleic Acids Res.* **27**, 2760–2769
6. Wankhade, S., Yu, Y., Weinberg, J., Tainsky, M. A., and Kannan, P. (2000) *J. Biol. Chem.* **275**, 29701–29708
7. Williams, T., and Tjian, R. (1991) *Genes Dev.* **5**, 670–682
8. Williams, T., and Tjian, R. (1991) *Science* **251**, 1067–1071
9. Higler-Eversheim, K., Moser, M., Schorle, H., and Buettner, R. (2000) *Gene (Amst.)* **260**, 1–12
10. Piao, Z., Lee, K. S., Kim, H., Peruchio, M., and Malakhosyan, S. (2000) *Genes Chromosomes Cancer* **30**, 113–122
11. Kannan, P., Buettner, R., Chiao, P. J., Yim, S. O., Sarkiss, M., and Tainsky, M. A. (1994) *Genes Dev.* **8**, 1258–1269
12. Somasundaram, K., Jayaraman, G., Williams, T., Moran, E., Frisch, S., and Thimmappaya, B. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 3088–3093
13. Anttila, M. A., Kellokoski, J. K., Moisio, K. I., Mitchell, P. J., Saarikoski, S., Syrjanen, K., and Kosma, V. M. (2000) *Br. J. Cancer* **82**, 1974–1983
14. Douglas, D. B., Akiyama, Y., Carraway, H., Belinsky, S. A., Esteller, M., Gabrielson, E., Weitzman, S., Williams, T., Herman, J. G., and Baylin, S. B. (2004) *Cancer Res.* **64**, 1611–1620
15. Gee, J. M., Robertson, J. F., Ellis, I. O., Nicholson, R. I., and Hurst, H. C. (1999) *J. Pathol.* **189**, 514–520
16. Heimberger, A. B., McGary, E. C., Suki, D., Ruiz, M., Wang, H., Fuller, G. N., and Bar-Eli, M. (2005) *Clin. Cancer Res.* **11**, 267–272
17. Jean, D., Gershenwald, J. E., Huang, S., Luca, M., Hudson, M. J., Tainsky, M. A., and Bar-Eli, M. (1998) *J. Biol. Chem.* **273**, 16501–16508
18. Ropponen, K. M., Kellokoski, J. K., Pirinen, R. T., Moisio, K. I., Eskelinen, M. J., Alhava, E. M., and Kosma, V. M. (2001) *J. Clin. Pathol.* **54**, 533–538
19. Ruiz, M., Pettaway, C., Song, R., Stoeltzing, O., Ellis, L., and Bar-Eli, M. (2004) *Cancer Res.* **64**, 631–638
20. Tellez, C., McCarty, M., Ruiz, M., and Bar-Eli, M. (2003) *J. Biol. Chem.* **278**, 46632–46642
21. Gershenwald, J. E., Sumner, W., Calderone, T., Wang, Z., Huang, S., and Bar-Eli, M. (2001) *Oncogene* **20**, 3363–3375
22. Zeng, Y. X., Somasundaram, K., and El-Deiry, W. S. (1997) *Nat. Genet.* **15**, 28–32
23. Karjalainen, J. M., Kellokoski, J. K., Eskelinen, M. J., Alhava, E. M., and Kosma, V. M. (1998) *J. Clin. Oncol.* **16**, 3584–3591
24. McPherson, L. A., Loktev, A. V., and Weigel, R. J. (2002) *J. Biol. Chem.* **277**, 45028–45033
25. Wajapeyee, N., and Somasundaram, K. (2003) *J. Biol. Chem.* **278**, 52093–52101
26. Wajapeyee, N., Raut, C. G., and Somasundaram, K. (2005) *Cancer Res.* **65**, 8628–8634
27. Yang, X. H., Sladek, T. L., Liu, X., Butler, B. R., Froelich, C. J., and Thor, A. D. (2001)

- Cancer Res.* **61**, 348–354
28. Zhang, L., Yu, J., Park, B. H., Kinzler, K. W., and Vogelstein, B. (2000) *Science* **290**, 989–992
 29. Lassus, P., Opitz-Araya, X., and Lazebnik, Y. (2002) *Science* **297**, 1352–1354
 30. Regl, G., Kasper, M., Schindler, H., Eichberger, T., Neill, G. W., Philpott, M. P., Esterbauer, H., Hauser-Kronberger, C., Frischauf, A. M., and Aberger, F. (2004) *Cancer Res.* **64**, 7724–7731
 31. Yamaguchi, H., Paranawithana, S. R., Lee, M. W., Huang, Z., Bhalla, K. N., and Wang, H. G. (2003) *Cancer Res.* **62**, 466–471
 32. Somasundaram, K., Reddy, S. P., Vinnakota, K., Britto, R., Subbarayan, M., Nambiar, S., Hebbar, A., Samuel, C., Shetty, M., Sreepathi, H. K., Santosh, V., Hegde, A. S., Hegde, S., Kondaiah, P., and Rao, M. R. (2005) *Oncogene* **24**, 7073–7083
 33. Cossarizza, A., Baccarani-Contri, M., Kalashnikova, G., and Franceschi, C. (1993) *Biochem. Biophys. Res. Commun.* **197**, 40–45
 34. MacLachlan, T. K., and El-Deiry, W. (2003) *Methods Mol. Biol.* **223**, 129–133
 35. Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000) *Cell* **102**, 33–42
 36. Green, D. R., and Kroemer, G. (2004) *Science* **305**, 626–629
 37. Burlacu, A. (2003) *J. Cell. Mol. Med.* **7**, 249–257
 38. Nechushtan, A., Smit, C. L., Hsu, Y. T., and Youle, R. J. (199) *EMBO J.* **18**, 2330–2341
 39. Li, M., Wang, Y., Hung, M. C., and Kannan, P. (2005) *Int. J. Cancer* **118**, 802–811
 40. Budihardjo, I., Oliver, H., Lutter, M., Luo, X., and Wang, X. (1999) *Annu. Rev. Cell Dev. Biol.* **15**, 269–290
 41. Jin, Z., and El-Deiry, W. S. (2005) *Cancer Biol. Ther.* **4**, 139–163
 42. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) *Cell* **91**, 479–489
 43. Lu, B., Mu, Y., Cao, C., Zeng, F., Schneider, S., Tan, J., Price, J., Chen, J., Freeman, M., and Hallahan, D. E. (2004) *Cancer Res.* **64**, 2840–2845
 44. Mirza, A., McGuiirk, M., Hockenberry, T. N., Wu, Q., Ashar, H., Black, S., Wen, S. F., Wang, L., Kirschmeier, P., Bishop, W. R., Nielsen, L. L., Pickett, C. B., and Liu, S. (2002) *Oncogene* **21**, 2613–2622
 45. Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B., and Korsmeyer, S. J. (2001) *Science* **292**, 727–730
 46. Chen, T. T., Wu, R. L., Castro-Munozledo, F., and Sun, T. T. (1997) *Mol. Cell. Biol.* **17**, 3056–3064
 47. Somasundam, K., and El-Deiry, W. S. (1997) *Oncogene* **14**, 1047–1057
 48. Imagawa, M., Chiu, R., and Karin, M. (1987) *Cell* **51**, 251–260
 49. Luscher, B., Mitchell, P. J., Williams, T., and Tjian, R. (1989) *Genes Dev.* **3**, 1507–1517
 50. Garcia, M. A., Campillos, M., Marina, A., Valdivieso, F., and Vazquez, J. (1999) *FEBS Lett.* **5**, 27–31
 51. Millward, T. A., Zolnierowicz, S., and Hemmings, B. A. (1999) *Trends Biochem. Sci.* **24**, 186–191
 52. Chen, J., Peterson, R. T., and Schreiber, S. L. (1998) *Biochem. Biophys. Res. Commun.* **247**, 827–832
 53. Murata, K., Wu, J., and Brautigan, D. L. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 10624–10629
 54. Marshall, C. J. (1991) *Trends Genet.* **7**, 91–95
 55. Forbes, I. J., Zalewski, P. D., Giannakis, C., and Cowled, P. A. (1992) *Exp. Cell Res.* **198**, 367–372
 56. Yu, G., Ahmad, S., Aquino, A., Fairchild, C. R., Trepel, J. B., Ohno, S., Suzuki, K., Tsuruo, T., Cowan, K. H., and Glazer, R. (1991) *Cancer Commun.* **3**, 181–188